Supplemental Figure Legends

Supplemental Figure 1

L1601PΔP, L1594PΔP, P12, and P12ΔP induced T-ALLs are mono- or oligoclonal. 20 μg of splenic DNA was extracted from L1601PΔP, L1594PΔP, P12, and P12ΔP tumors, digested with EcoRI, run in a 0.8% agarose gel and then analyzed on a Southern blot hybridized to an IRES probe. EcoRI cuts only once in the vector sequence 5' to the IRES probe, so each EcoR1-digested proviral integrant produces a different sized DNA fragment (asterisks). Numbers correspond to kilobase pairs. Representative samples are shown. BL/6 is wildtype C57BL/6 spleen.

Supplemental Figure 2

Notch1-mediated and K-ras^{G12D}/Notch1-mediated T-ALLs show activation of the Ras/MAPK pathway. T-ALL infiltrates in the liver induced by Notch1 mutants **(A)** and K-ras^{G12D}/Notch1 mutants **(B)** were stained with antibodies specific for phospho-ERK using an immunoperoxidase staining method that produces a brown color (hematoxylin counterstain).

Supplemental Figure 3

Notch1-mediated T-ALLs show activation of the Ras/MAPK pathway. Extracts of tumor samples from L1601PΔP and P12ΔP mice were analyzed on Western blots stained with phospho-ERK (P-ERK) and ERK (t-ERK) antibodies. Extracts from cells treated with the MAPK inhibitor U0126 for 2 hours were included as a control.

Supplemental Figure 4

Analysis of proviral integration sites and clonality of tumors generated in LCR mice. (A) L1601P, N1ΔP, and L1601PΔP T-ALLs arising in the LCR (K-ras^{G12D}-expressing) background have intact proviral integrants. 20 μg of DNA was extracted from the indicated L1601P and L1601PΔP tumors, digested with EcoRV, run in a 0.8% agarose gel and then analyzed on a Southern blot hybridized to an IRES probe. EcoRV cuts in the vector sequence once 5' to the inserted cDNA and once 3' to the inserted cDNA and IRES sequence, generating a ~10.8 Kb fragment from intact L1601P proviruses and a ~9.7 kb fragment from intact L1601PΔP proviruses. **(B)** L1601P, N1ΔP, and L1601PΔP tumors on the LCR (K-ras^{G12D}-expressing) background are mono- or oligoclonal. 20 μg of DNA was extracted from indicated L1601P and L1601PΔP tumors, digested with EcoRI, run on a 0.8% agarose gel and then analyzed on a Southern blot hybridized to an IRES probe. EcoRI cuts in the proviral sequence 5' to the IRES probe and again in the genomic sequence at the integration site. Each band (asterisk) represents a unique integration site. Numbers correspond to kilobase pairs. Representative samples are shown.

Supplemental Figure 5

Flow cytometric analysis of K-ras^{G12D}/Notch T-ALLs. Splenocytes harvested from moribund, leukemic mice were depleted of red cells and analyzed for GFP expression and CD4/CD8 profile by flow cytometry. Four samples of each type of tumor are shown.

Supplemental Figure 6

MigR1, L1601P, N1ΔP, and L1601PΔP tumors show excision of the floxed stop cassette by cre recombinase. PCR was performed on genomic DNA extracted from the indicated tumors using primers specific for K-ras intron 1 and the original targeting vector and run in a 2% agarose gel. The looped out stop cassette product is 325 bp and the wildtype product is 285 bp. BL/6 is wildtype C57BL/6 spleen. Representative samples are shown.

Supplemental Figure 7

Immunohistochemistry of Kras/MigR1 and Kras/N1ΔP tumors. T-ALL infiltrates in the liver induced by K-ras^{G12D}-positive BM progenitors transduced with MigRI or the Notch1ΔP allele were stained with antibodies specific for CD3, terminal deoxytransferase (TdT), and Notch1, using an immunoperoxidase method that produces a brown color (hematoxylin counterstain).

Supplemental Figure 8

K-ras^{G12D} does not increase Notch activity. (**A)** 8946 cells were co-transduced with a GFP$^+$ vector (MigR1, L1601P, or L1601PΔP) and an NGFR$^+$ vector (MSCV-IRES-NGFR or MSCV-Kras^{G12D}-IRES-NGFR) in a 3x2 matrix format. Two days later, cells were divided in triplicate, analyzed for GFP$^+$/NGFR$^+$ cells, and then treated with carrier control or doxycycline. 6 days later the cells were again analyzed for GFP$^+$/NGFR$^+$ cells. Fold increase of GFP$^+$/NGFR$^+$ cells on Day 6 relative to the Day 0 was determined for each retrovirus combination. **(B)** Two days after transduction, GFP$^+$/NGFR$^+$ 8946 cells were sorted and rested for 5 days before treatment with doxycycline. After 23 hours of treatment, RNA was harvested and assayed for c-*Myc* and *Deltex1* expression by real-time PCR. **(C)** Western blot showing ERK phosphorylation in 8946 cells in the presence of the K-ras^{G12D} allele.

Supplemental Figure 9.

Immunohistochemical analysis of Notch1 expression in T-ALLs induced by K-ras^{G12D}-positive BM progenitors transduced with MigRI, L1601P or L1601PΔP. Paraffin-embedded sections of liver were stained with an antibody specific for Notch1 using an immunoperoxidase method that produces a brown color (hematoxylin counterstain).

Supplemental Figure 10.

Immunohistochemical analysis of Notch1 expression in T-ALLs induced by various Notch1 gain-of-function alleles. Paraffin-embedded sections of liver were stained with an antibody specific for Notch1 using an immunoperoxidase method that produces a brown color (hematoxylin counterstain).

Supplemental Figure 11.

Comparison of retroviral and endogenous Notch1 expression in K-ras^{G12D}/N1ΔP tumors. Extracts prepared from spleens involved by T-ALL were analyzed on a Western blot stained with a Notch1 antibody that recognizes the intracellular domain. An extract from 293T cells transfected with MigRI-N1ΔP was included as a positive control and size marker. High molecular weight cross-reactive polypeptides represent unprocessed full-length Notch1 and N1ΔP, which are not resolved on this gel. N^{TM}, N^{TM}ΔP; Notch transmembrane subunits produced by furin processing of Notch1 and N1ΔP, respectively.

Supplemental Figure 12.

Sensitivity of Kras^{G12D}/Notch tumors to PI3K and MAPK inhibition. Primary cells cultured from tumors that developed in mice reconstituted with K-ras^{G12D}/L1601P or K-ras^{G12D}/L1601PΔP BM cells were treated with DMSO carrier, 20 μM LY294002 (PI3K inhibitor), or 10 μM U0126 in triplicate. Fold cell growth (relative to initial cell number) was assessed after 6 days. *No cells detected.

Supplemental methods

Plasmids. The K-ras^{G12D} cDNA was obtained as a gift from David Tuveson and was cloned into MSCV-IRES-NGFR.

Cell culture. The MAPK inhibitor U0126 was obtained from Cell Signaling Technology; and the PI3K inhibitor LY294002 was obtained from Calbiochem.

Southern blotting. High-molecular-weight DNA was isolated from fresh or snap-frozen spleen tissue. DNA (20 µg) was digested with the appropriate restriction enzymes, fractionated by electrophoresis, and blotted overnight onto Nytran membrane (Schleicher & Schuell, Keene, NH) via alkaline transfer. Blots were hybridized with gel-purified ^{32}P-labeled probes corresponding to the IRES fragments of MigR1.

Western blotting and histology. Phospho-ERK and total-ERK were detected using rabbit polyclonal antibodies (Cell Signaling Technology). Sections of formalin-fixed paraffin embedded mouse spleen, liver, thymus, lung, kidney and BM were stained with hematoxylin and eosin. Immunohistochemistry was performed using 4 um thick formalin-fixed, paraffin-embedded tissue sections. Briefly, slides were soaked in xylene, passed through graded alcohols and put in distilled water. Slides were then pre-treated with 1.0 mM EDTA, pH 8.0 (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) per the manufacturer's instructions followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with

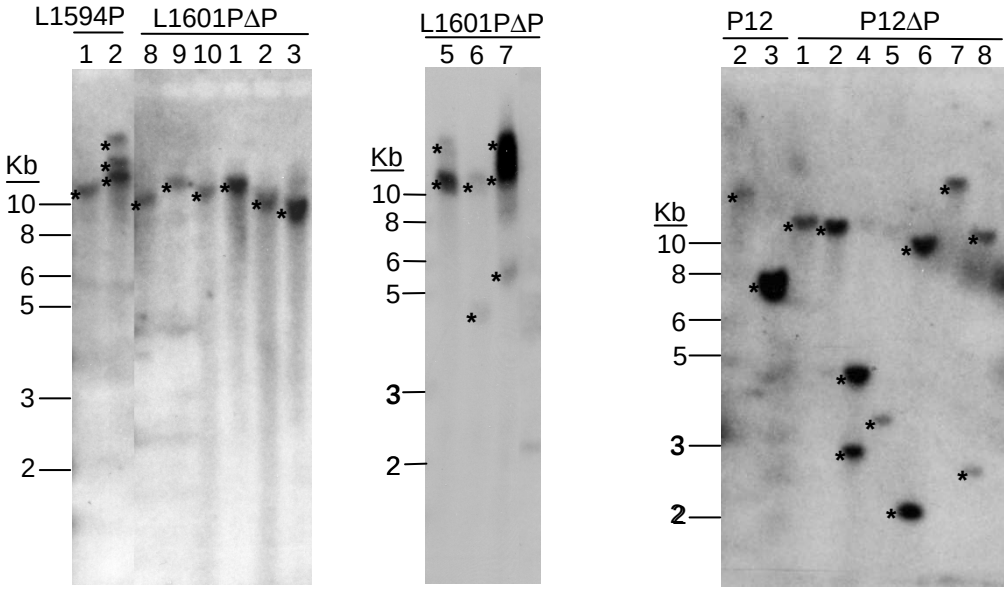
Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity. For Notch1, a polyclonal rabbit antibody against the transcriptional activation domain of Notch1 (1) was applied at 1:1500 in DAKO diluent for 1 hour. Slides were washed in 50 mM Tris-Cl, pH 7.4, and detected with anti-rabbit Envision+ kit (DAKO) per the manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin.

DNA sequencing. To amplify the proviral sequences encoding the LNRs, HD, and transmembrane regions of Notch1 from tumor DNAs, the following primers were used: forward, 5'-CCACATCCTGGACTACAGCTTC-3'; reverse, 5'-CAGACACTTTGAAGCCCTCAG-3'. Products of the correct size (1,054 bp) were gel-excised and cloned into pCR2.1. Plasmids were confirmed to have the correct inserts by digestion with EcoRI and sequenced bidirectionally. Sequences were aligned and analyzed with Mutation Surveyor software (Softgenetics, State College, PA).

Supplemental reference

1. Chiang, M.Y., Xu, M.L., Histen, G., Shestova, O., Roy, M., Nam, Y., Blacklow, S.C., Sacks, D.B., Pear, W.S., and Aster, J.C. 2006. Identification of a conserved negative regulatory sequence that influences the leukemogenic activity of NOTCH1. *Mol Cell Biol* 26:6261-6271.

Supplemental Figure 1



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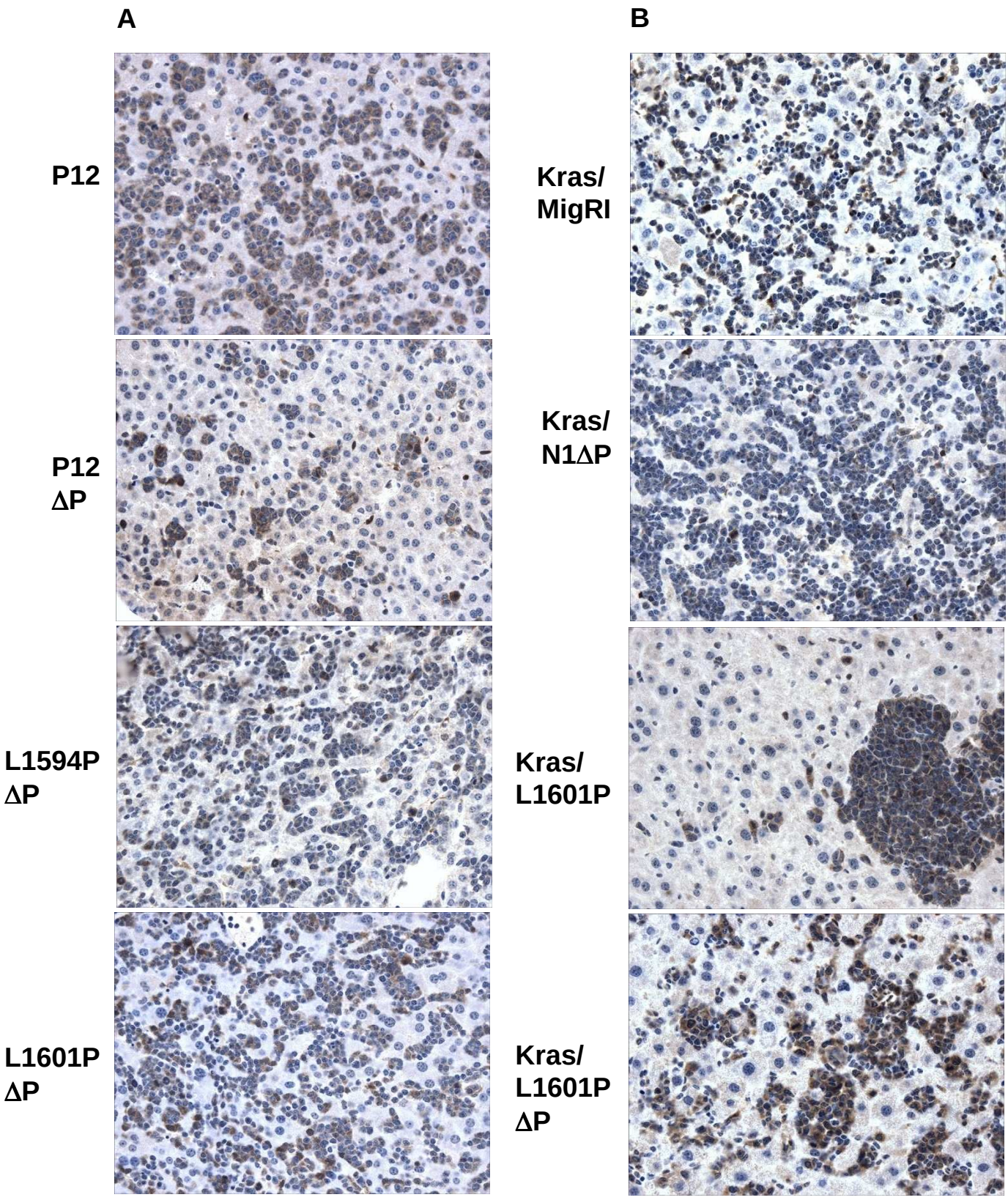

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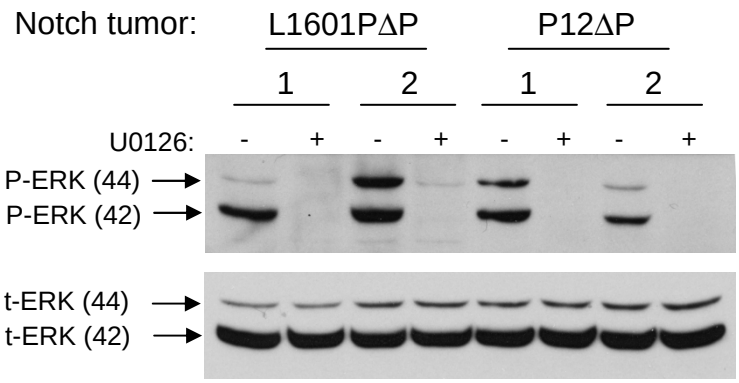

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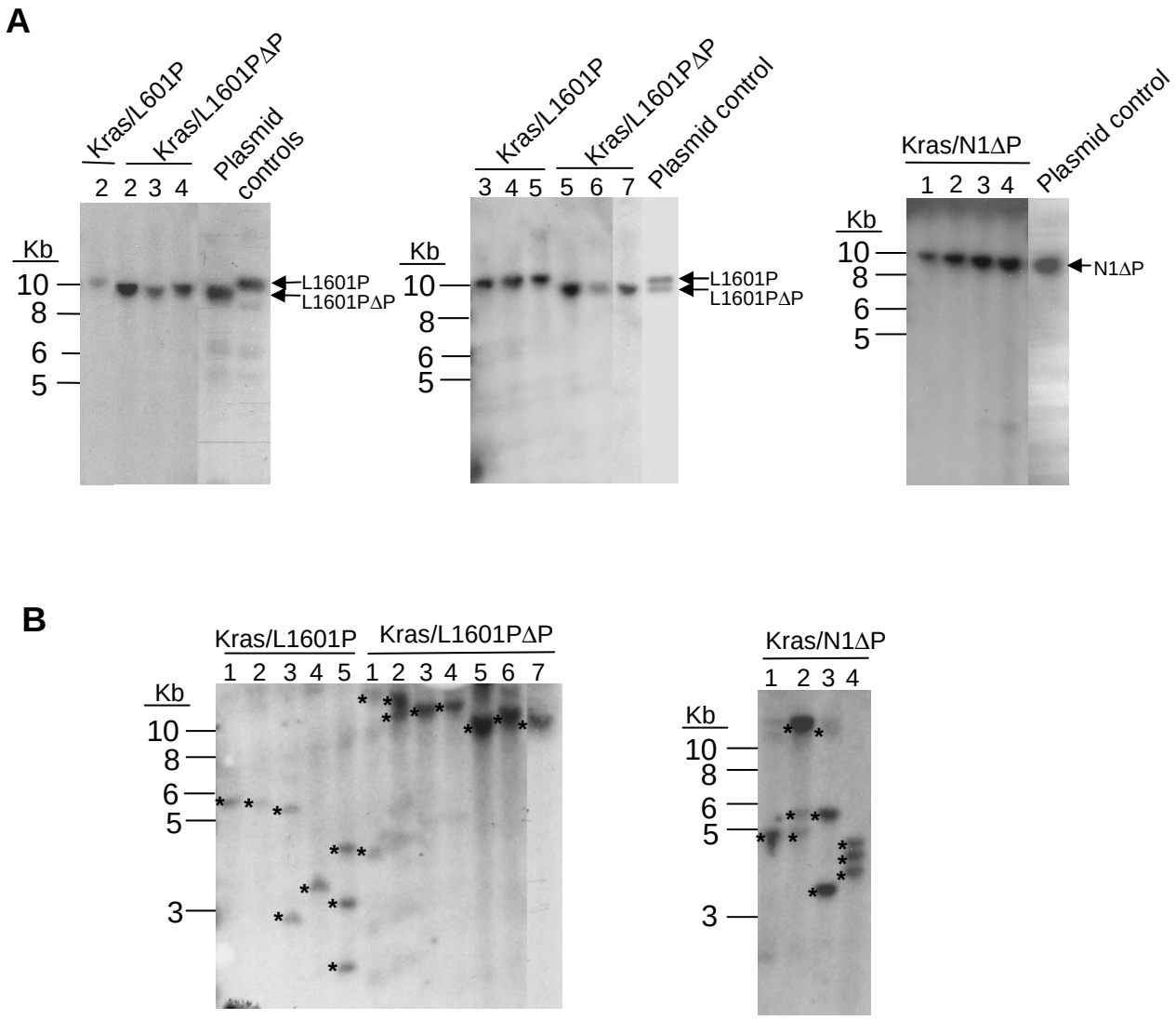

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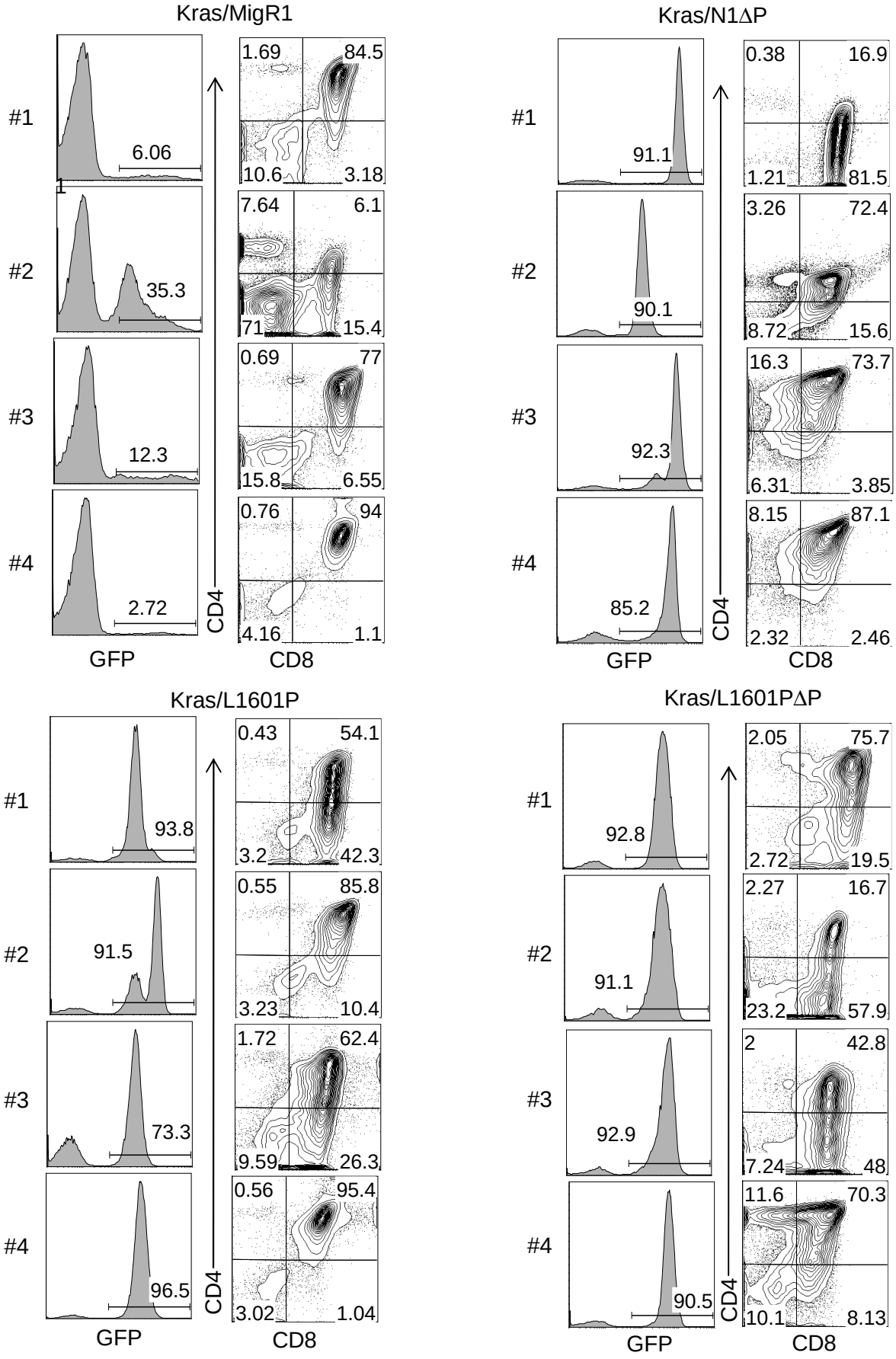

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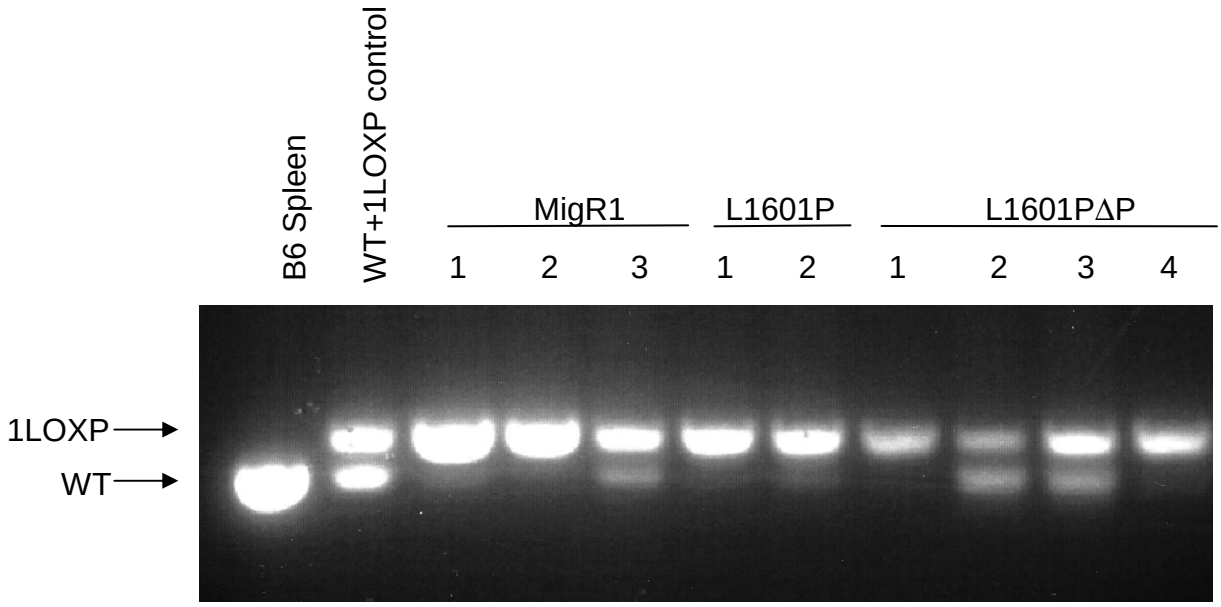

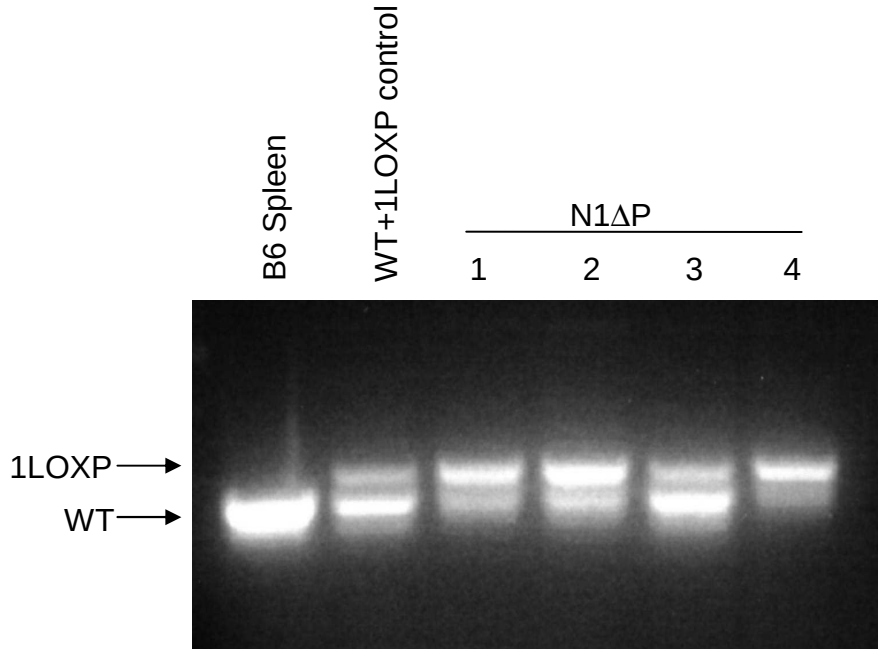

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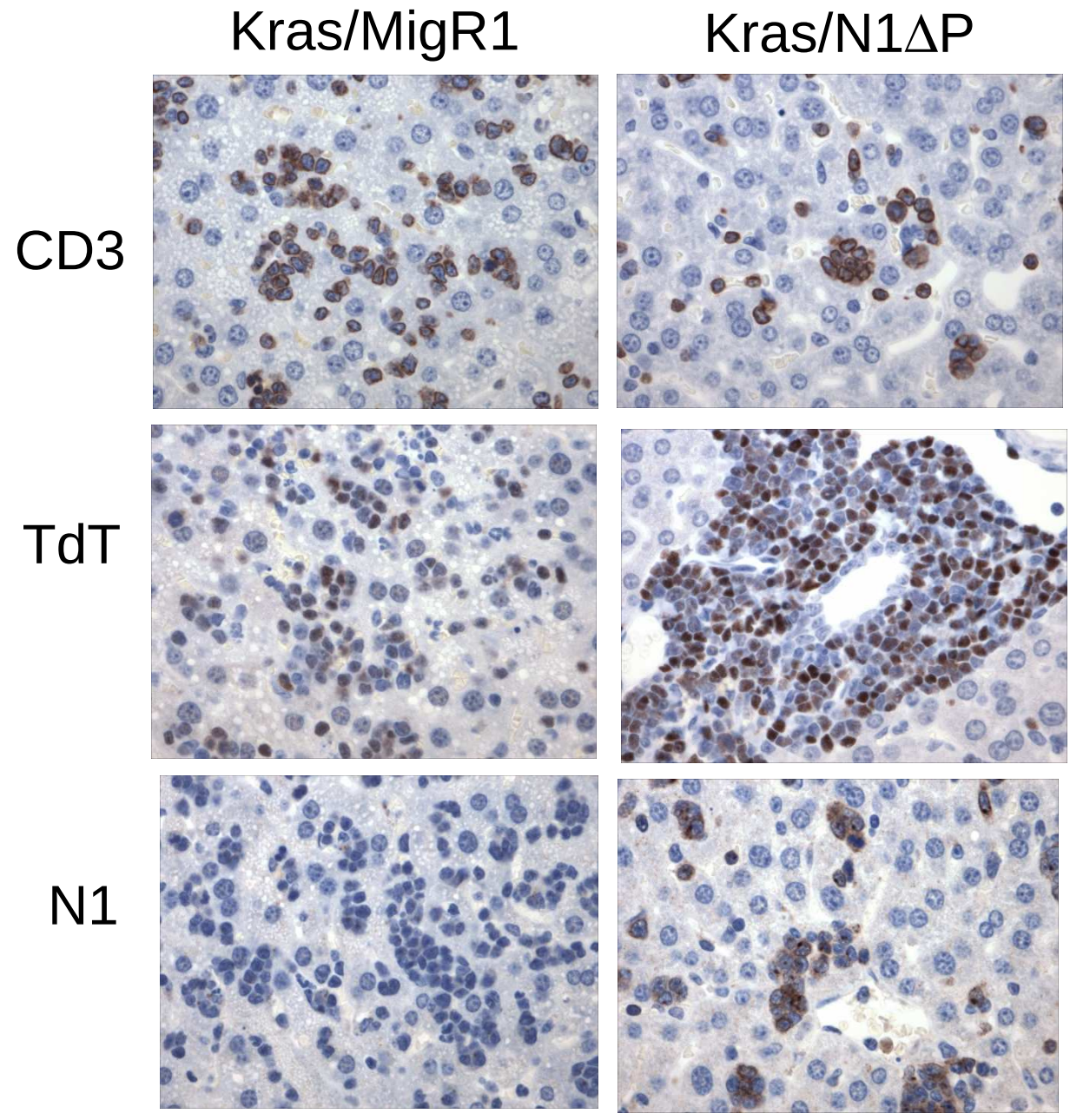

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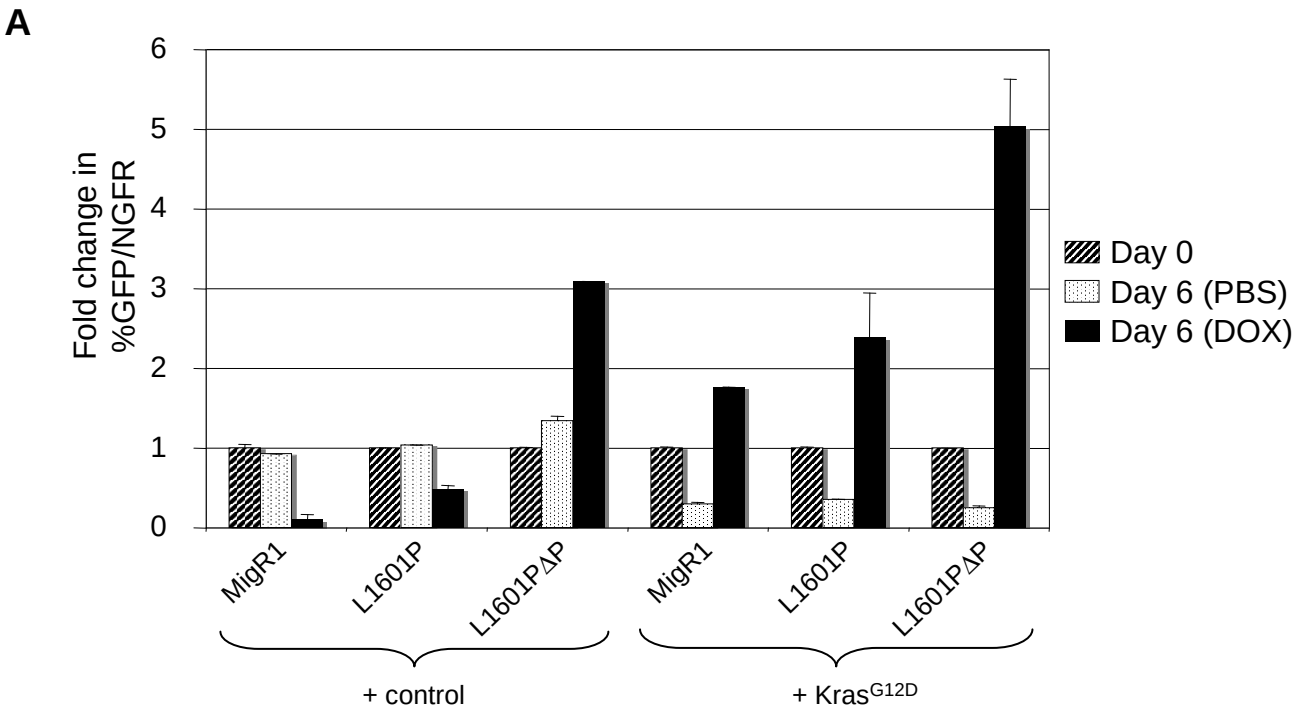
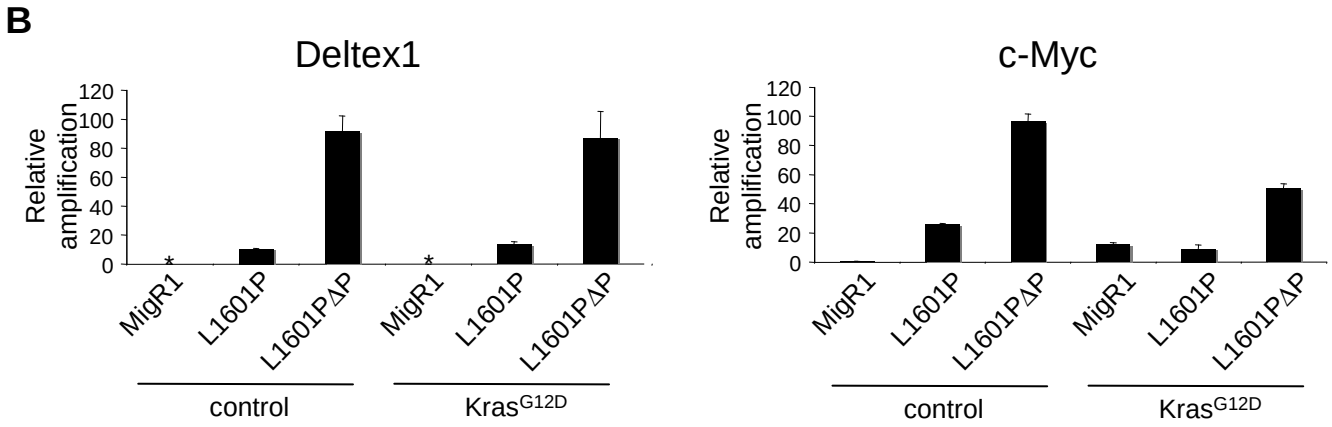
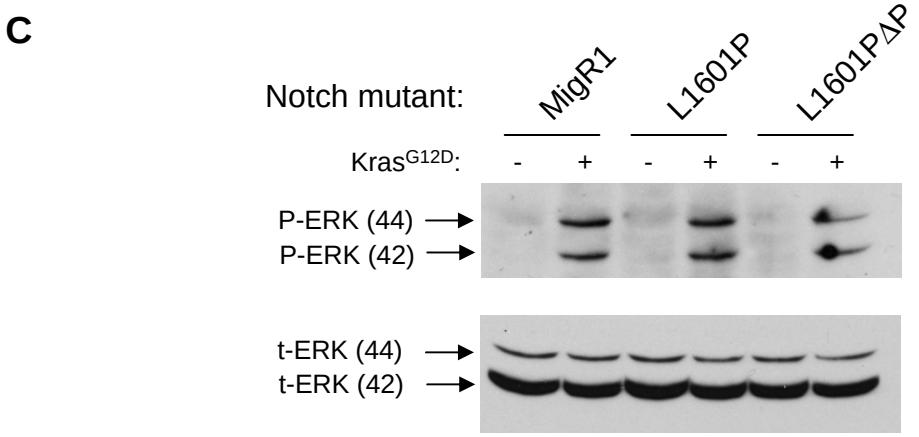
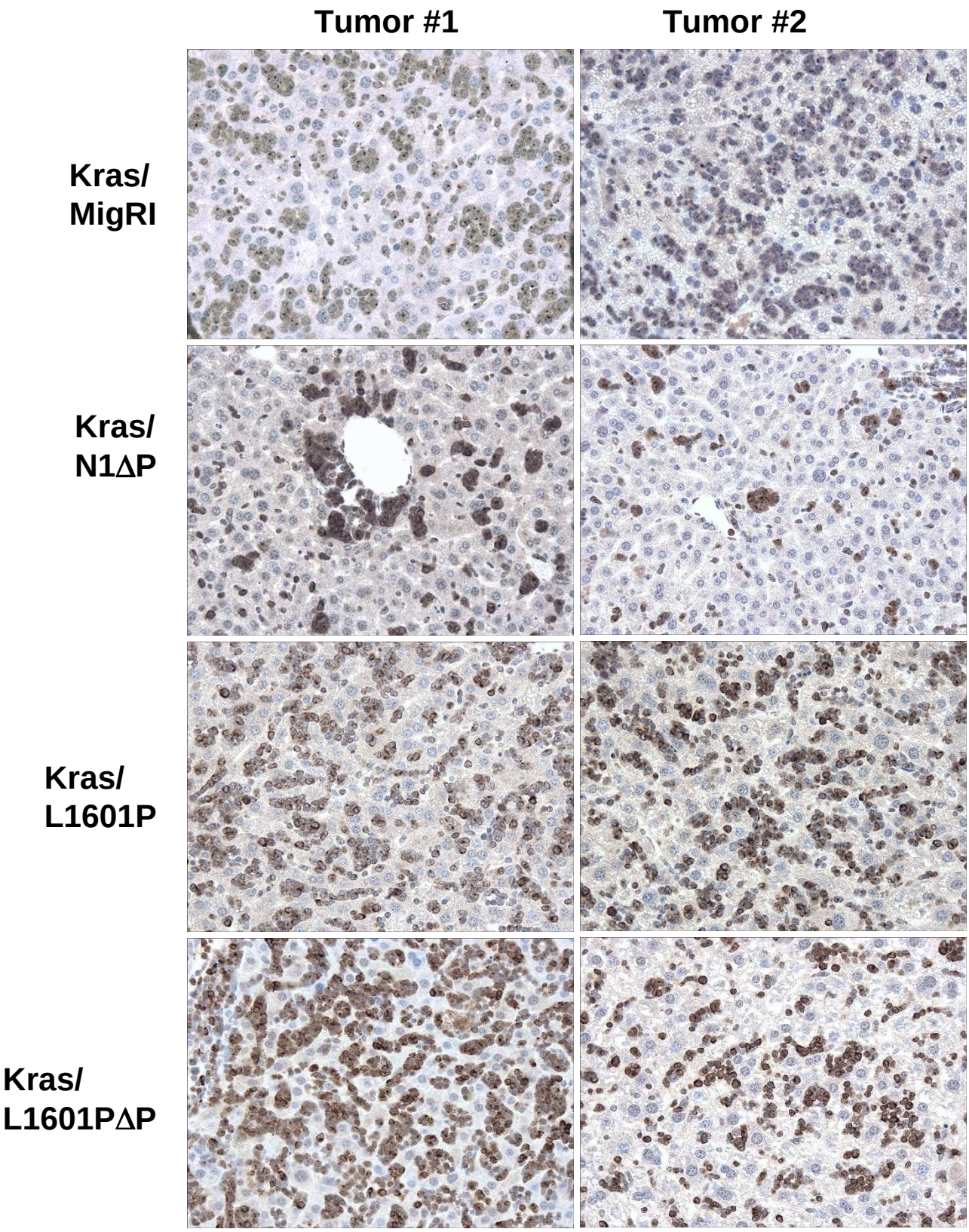






Supplemental Figure 9



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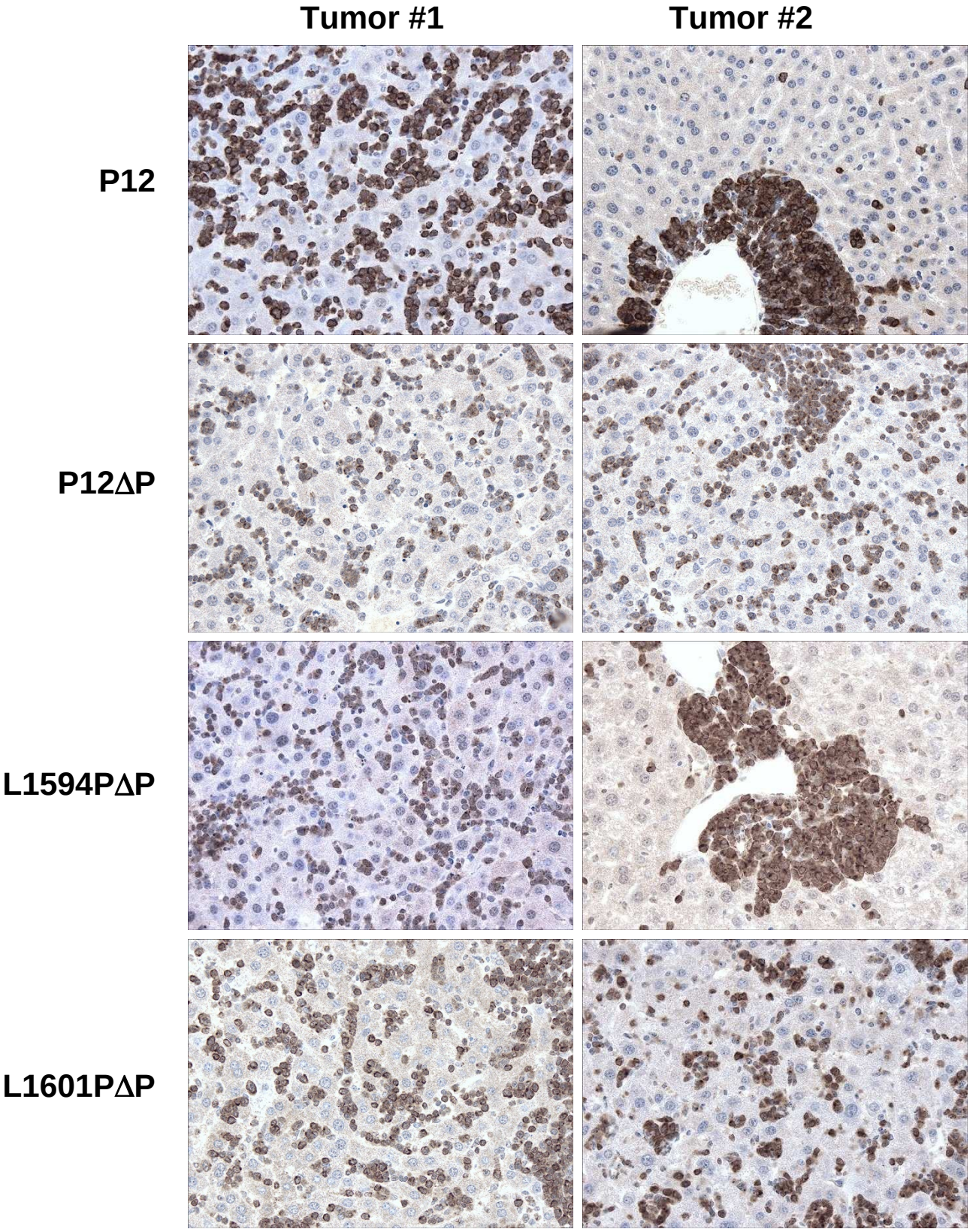

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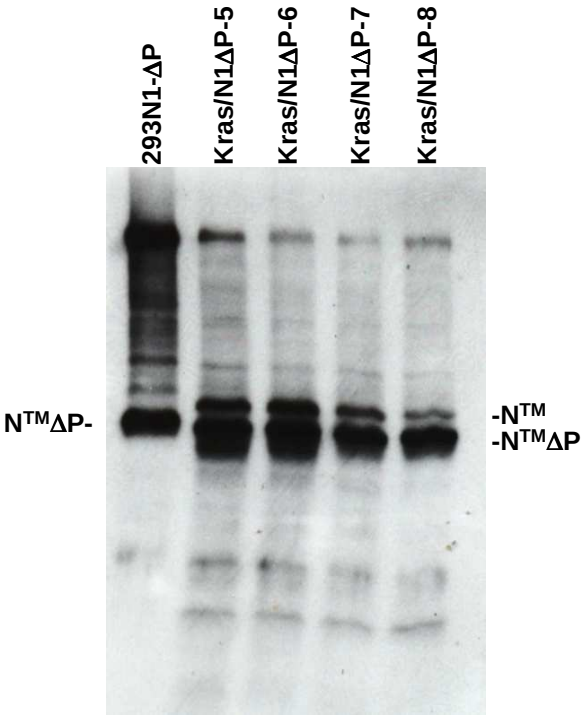

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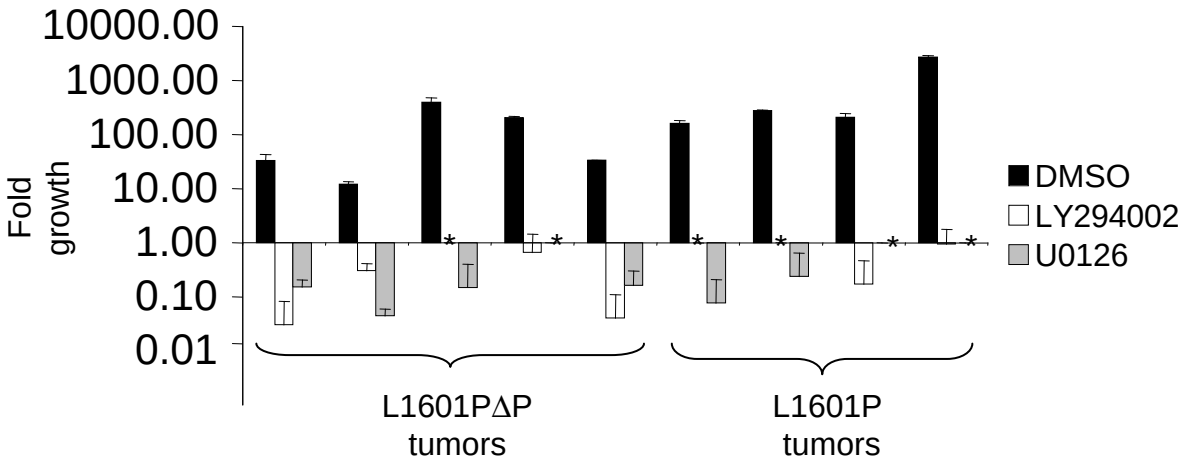

Supplemental Table 1. WBC, Spleen Weights, Median Onset of T-ALL

Notch1 Allele	# Tumors Analyzed[A]	Mean WBC (k/mcl)[B]	WBC Range (k/mcl)	Mean Spleen Wt (mg) [C]	Spleen Wt Range (mg)	Median Leukemic Onset (days)[D]	Leukemic Onset Range (days)
ICN1	6	51	6-193	835	364-1271	66	48-87
P12	3	40	5-104	266	75-401	121	91-125
L1601PΔP	13	31	9-120	467	117-1020	113	61-189
L1594PΔP	2	86	19-154	351	188-513	101	68-134
P12ΔP	7	41	11-88	583	438-858	90	75-127
Kras/MigR1	5	28	8-89	306	207-363	84	72-131
Kras/L1601P	5	174	99-300	568	422-743	72	67-76
Kras/L1601PΔP	7	252	21-825	596	350-1012	53	42-59
Kras/N1ΔP	4	20	12-28	199	130-311	95	80-107

[A]Tumors for which spleen and WBC data were determined. [B]Normal WBC is approximately 2.9-11.9 k/mcl. [C]Normal spleen wt is approximately 66-148 mg. [D]Onset of morbidity from tumor burden after transplant